

RESEARCH PAPER

Rubisco activase and wheat productivity under heat-stress conditions

Zoran Ristic^{1,*}, Ivana Momčilović^{2,†}, Urška Bukovnik^{2,‡}, P. V. Vara Prasad², Jianming Fu², Benjamin P. DeRidder³, Thomas E. Elthon⁴ and Novica Mladenov⁵

¹ United States Department of Agriculture–Agricultural Research Service, Plant Science and Entomology Research Unit, 4008 Throckmorton Hall, Kansas State University, Manhattan, KS 66506, USA

² Department of Agronomy, Kansas State University, Manhattan, KS 66506, USA

³ Department of Biology, Grinnell College, Grinnell, IA 50112, USA

⁴ Department of Agronomy and Horticulture, Plant Science Initiative, Center for Biotechnology, and School of Biological Sciences, University of Nebraska, Lincoln, NE 68588, USA

⁵ Institute of Field and Vegetable Crops, Novi Sad, Serbia

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Abstract

Rubisco activase (RCA) constrains the photosynthetic potential of plants at high temperatures (heat stress). Endogenous levels of RCA could serve as an important determinant of plant productivity under heat-stress conditions. Thus, in this study, the possible relationship between expression levels of RCA and plant yield in 11 European cultivars of winter wheat following prolonged exposure to heat stress was investigated. In addition, the effect of a short-term heat stress on RCA expression in four genotypes of wheat, five genotypes of maize, and one genotype of *Arabidopsis thaliana* was examined. Immunoblots prepared from leaf protein extracts from control plants showed three RCA cross-reacting bands in wheat and two RCA cross-reacting bands in maize and *Arabidopsis*. The molecular mass of the observed bands was in the range between 40 kDa and 46 kDa. Heat stress affected RCA expression in a few genotypes of wheat and maize but not in *Arabidopsis*. In wheat, heat stress slightly modulated the relative amounts of RCA in some cultivars. In maize, heat stress did not seem to affect the existing RCA isoforms (40 kDa and 43 kDa) but induced the accumulation of a new putative RCA of 45–46 kDa. The new putative 45–46 kDa RCA was not seen in a genotype of maize (ZPL 389) that has been shown to display an exceptional sensitivity to heat stress. A significant, positive, linear correlation was found between the expression of wheat 45–46 kDa RCA and plant productivity under heat-stress conditions. Results support the hypothesis that endogenous levels of RCA could play an important role in plant productivity under supraoptimal temperature conditions.

Key words: Heat stress, heat tolerance, Rubisco activase, wheat productivity.

Introduction

Rubisco activase (RCA) is a nuclear-encoded, cytosol-synthesized chloroplast protein that regulates activity of Rubisco by promoting the ATP-dependent removal of inhibitory sugar phosphates from Rubisco active sites (Portis, 1995; Salvucci and Ogren, 1996). This action of

RCA is essential for photosynthesis because it frees the active site of Rubisco for spontaneous carbamylation by CO₂ (Portis, 1995; Salvucci and Ogren, 1996; Spreitzer and Salvucci, 2002). In most species studied, RCA is found in two isoforms, the longer α (43–47 kDa) and the shorter β

* To whom correspondence should be addressed: E-mail: zoran.ristic@ars.usda.gov

† Present address: Department of Plant Physiology, Institute for Biological Research, 11060 Belgrade, Serbia.

‡ Present address: Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA.

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(41–42 kDa) (Salvucci *et al.*, 1987), both of which are capable of catalysing ATP hydrolysis and Rubisco activation (Shen *et al.*, 1991). The two RCA isoforms have a similar primary structure, except the longer isoform has an additional 27–36 amino acids at the C-terminal end (Werneke *et al.*, 1989; Rundle and Zielinski, 1991; To *et al.*, 1999). Some studies have shown that the number of RCA cross-reacting polypeptides may be fewer or greater than two under optimal growth conditions. In maize (*Zea mays* L.), for example, Sánchez de Jiménez *et al.* (1995) observed only one RCA isoform; and in barley (*Hordeum vulgare* L.), Rundle and Zielinski (1991) reported three RCA polypeptides. The number of RCA genes is also variable. In dicots, RCA is encoded by one (Werneke *et al.*, 1988) to five (Qian and Rodermel, 1993) and in monocots by two genes (Rundle and Zielinski, 1991; Ayala-Ochoa *et al.*, 2004; Zhang and Komatsu, 2000). Alternative splicing of a single pre-mRNA (Werneke *et al.*, 1989; Rundle and Zielinski, 1991; To *et al.*, 1999) or the expression of different genes without this regulation (Salvucci *et al.*, 2003) can account for the appearance of multiple forms of RCA.

The role of RCA as Rubisco's chaperone (Portis, 2003) becomes most apparent during periods of heat stress, when Rubisco deactivation accelerates and photosynthesis is inhibited. Because of its low temperature optimum for ATPase activity (Salvucci and Crafts-Brandner, 2004a) and an inherent thermal instability (Salvucci *et al.*, 2001; Salvucci and Crafts-Brandner, 2004b), RCA is unable to keep pace with the rate of Rubisco deactivation during periods of moderate heat stress. Recent studies indicate that expression and endogenous levels of RCA can be modulated by heat stress. In maize, Sánchez de Jiménez *et al.* (1995) observed that heat stress increased the accumulation of a RCA isoform of 41 kDa and induced the appearance of a novel putative isoform of 43 kDa. Similarly, in wheat (*Triticum aestivum* L.), Law and Crafts-Brandner (2001) reported that heat stress increased the levels of the shorter RCA isoform of 42 kDa (β) and induced the accumulation of a new putative isoform of 41 kDa. Also, in cotton (*Gossypium hirsutum* L.), Law *et al.* (2001) detected a new heat-induced form of RCA at 46 kDa. It is thought that heat-induced changes in activase protein expression, whether the appearance of new isoforms or fluctuations in the levels of constitutively-expressed RCA proteins, may alleviate the inhibitory effect of heat stress on photosynthesis (Law and Crafts-Brandner, 2001). However, neither the catalytic characteristics nor thermal stability of heat-induced RCA isoforms have been investigated because none have been successfully isolated to date.

Nevertheless, studies have shown that endogenous levels of RCA are of importance to plant photosynthesis under both optimal and supraoptimal temperatures. In tobacco (*Nicotiana tabacum* L.), for example, RCA-deficient plants had reduced rates of CO₂ fixation at 25 °C (Mate *et al.*, 1993) and increased heat sensitivity of photosynthesis (Sharkey *et al.*, 2001) compared with plants with normal levels of RCA. Similarly, in *Arabidopsis* and

Antarctic hairgrass (*Deschampsia antarctica* L.), transgenic plants expressing suboptimal levels of RCA displayed a greater reduction of photosynthesis under moderately high temperatures compared with wild-type plants (Salvucci *et al.*, 2006). Because heat-induced reductions in photosynthetic performance are thought to contribute to substantial crop losses (Quinn and Williams, 1985), these results suggest that endogenous levels of RCA may be a limiting factor that constrains plant yield under supraoptimal temperatures. To date, the only evidence supporting this hypothesis was reported by Martínez-Barajas *et al.* (1997), who examined grain yield and levels of RCA in two genetically related populations of the same maize cultivar under field conditions. They observed that the high-yielding population had higher levels of RCA than the low-yielding population. It is not clear, however, whether plants of the two maize populations in their study had experienced heat stress.

Endogenous levels of RCA could serve as an important determinant of plant productivity under elevated temperatures. To investigate this hypothesis further, expression levels of RCA and plant yield in 11 European cultivars of winter wheat following prolonged exposure to heat stress were examined. In addition, the effect of short-term heat stress on RCA expression in several genotypes of wheat and maize and one genotype of *Arabidopsis thaliana* was investigated.

Materials and methods

Plant material and experimental conditions

RCA expression levels and plant yield in winter wheat following exposure to prolonged heat stress: Expression levels of RCA and plant yield were investigated in mature plants (flowering stage) of 11 cultivars of winter wheat. Mature plants were used because, under field conditions, wheat is more likely to encounter prolonged exposure to elevated temperature at flowering stage. Growth conditions and heat-stress treatment were similar to those described by Ristic *et al.* (2008). Briefly, seeds were germinated in trays containing potting soil (Metro Mix 200; Hummert Intl., Topeka, KS) in a greenhouse. Ten-day-old seedlings were vernalized at 4 °C for 8 weeks, after which they were transplanted into 10 pots (three seedlings per pot; pot diameter at the top and the bottom was 21 cm and 16 cm, respectively; pot height was 20 cm) containing Metro Mix 200 potting soil. Plants were grown in a greenhouse and watered daily in the winter of 2006. Miracle-Gro fertilizer (24:8:16; Stern's Miracle-Gro Products, Inc., Port Washington, NY) was applied (according to the manufacturer's instructions) weekly during the entire duration of the experiment. At flowering stage [growth stage Feekes 10.5.1 (Large, 1954)], plants of each cultivar were divided into control (five pots) and heat-stress (five pots) groups. The control group was maintained under growth conditions in a greenhouse, and the treatment group was exposed to

36/30 °C (day/night) heat stress for 16 d [90–100%; 16/8 h photoperiod; 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF (Sylvania cool white fluorescent lamps)] in a growth chamber (Conviron, PGW-36, Winnipeg, MB, Canada). The heat treatment was administered by a gradual increase of the temperature from ambient to 36 °C over 1 h. Air temperature, RH, and light level were continuously monitored in the growth chamber and, in the greenhouse, air temperature was measured at hourly intervals. Average daily temperature in the greenhouse was 22.7 ± 2.8 °C during the period of heat-stress treatment. The PPF and RH in the greenhouse ranged from 270 to 320 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (16 h photoperiod; supplementary light was used to extend daylight period) and 55–70%, respectively. To minimize or avoid possible dehydration of the leaf tissue during heat treatment, pots of the treatment and control group were kept in trays containing shallow water (~1 cm). Following long-term heat treatment, plants were transferred to a greenhouse and allowed to recover at ambient temperature until maturity. At maturity, shoots (stem, leaves, and ears) of all plants except those used for RCA analysis were harvested (cut at the soil surface), and dry mass of the shoots was measured after oven-drying at 60 °C for 7 d. Expression levels of RCA were determined after 7 d of heat stress. For RCA analysis, samples of leaf tissue were obtained from the flag leaf blades from two randomly selected plants (each plant was taken from a different replicate pot) from both control and heat-stressed groups. Collected leaves were immediately frozen in liquid nitrogen and stored at –80 °C until further use.

Grain yield under field conditions: This study included data on grain yield encompassing a period of 11 years (from 1996 to 2006) and related these data to expression levels of RCA that were determined in the experiment described above. Plants of each cultivar were grown in the experimental field of the Institute of Field and Vegetable Crops, Rimski Šančevi, Novi Sad, Serbia (45°33' N, 19°85' E, 82 m altitude). Previous crops were pea (*Pisum sativum* L.) or soybean (*Glycine max* L.). The soil type was Chernozem Chernic (FAO, 1998). Study areas were plowed and disc-harrowed to prepare the seed beds. Fertilizer (NPK, 15:15:15, 250 kg ha⁻¹) was applied each year prior to planting. Plants were grown in a randomized complete block design with four replications. Plots of 5 m² with 10 rows spaced 10 cm apart were seeded (550 kernels m⁻²) between October 5 and October 15. At the end of March or the beginning of April each year, plants were fertilized [150–250 kg ha⁻¹ of calcium ammonium nitrate (27% N)] according to soil test results. Fields were rain-fed, and no supplemental irrigation was provided. Weeds and insects were controlled post-emergence with commercial formulations of GRANSTAR-75 (tribenuron-methyl; 23 g ha⁻¹) and DECIS 2.5-EC (deltamethrin; 0.2–0.3 l ha⁻¹), respectively. Grains were harvested with a combine harvester (Wintersteiger, Austria), and grain yield was measured and expressed in tons per hectare at 13% moisture content. For

each cultivar, grain yield encompassing a period of 11 years was averaged (average grain yield). Data on average grain yield of all cultivars were then related to expression levels of RCA (see Data analysis section).

RCA expression in wheat, maize, and Arabidopsis following exposure to a short-term heat stress: Effect of a brief heat stress on RCA expression was examined in winter wheat cultivars Ventnor and Karl-92 (Yang *et al.*, 2002); spring wheat cultivars Seri-82 and Pavon-76 (Singh *et al.*, 2001); maize genotypes ZPBL 1304, ZPL 389 (Ristic and Cass, 1993), Zmeftu-wt, and Zmeftu::mum540 (Ristic *et al.*, 2004); commercial maize line B-73; and *Arabidopsis thaliana* (ecotype Col-0). In wheat, RCA expression was investigated in mature plants at flowering stage [growth stage Feekes 10.5.1 (Large, 1954)], and in maize and *Arabidopsis*, RCA expression was examined in 3-week-old plants. Wheat plants were grown in four pots under conditions similar to those described previously. At the beginning of flowering, plants were divided into control (two pots) and heat-stress (two pots) groups. The control group was maintained under growth conditions in a greenhouse, and the treatment group was exposed to 40 °C for 4 h [90–100% RH; 16 h photoperiod; 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF (Sylvania cool white fluorescent lamps)] in a growth chamber. The temperature was gradually increased from 22 °C to 40 °C over 1 h. Exposure time for heat-stress treatment started when the temperature reached 40 °C. During the heat-stress treatment, pots of both control and heat-stress groups were kept in trays containing shallow water (~1 cm). For RCA analysis, samples of flag leaf tissue were collected from two plants (each plant was taken from a different pot) from both control and heat-stressed groups. Collected samples were immediately frozen in liquid nitrogen and stored at –80 °C until further use.

In maize and *Arabidopsis*, RCA expression was investigated in two independent experiments. In each experiment, plants of each genotype of both maize and *Arabidopsis* were grown in four pots in a growth chamber [22/17 °C (day/night); 70% RH; 16/8 h photoperiod; 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF (Sylvania cool white fluorescent lamps)], watered daily, and fertilized weekly as described previously. Three-week-old plants of both maize and *Arabidopsis* were divided into control (two pots) and heat-stress (two pots) groups. The control group was maintained under growth conditions in the growth chamber, and the heat-stress group was exposed to heat stress in a growth chamber. Maize was exposed to 45 °C for 4 h, and *Arabidopsis* was exposed to 35 °C for 24 h. The heat treatment was characterized by a gradual increase in temperature from 22 °C to heat-stress temperature (45 °C for maize and 35 °C for *Arabidopsis*) over 1 h. To minimize or avoid possible dehydration of the leaf tissue during heat treatment, pots of both control and heat-stress groups were kept in trays containing shallow water. For RCA analysis in maize, leaf tissue (the youngest fully expanded leaf) was collected from both control and heat-stressed plants immediately after the 4 h heat treatment; and

for RCA analysis in *Arabidopsis*, leaf tissue was collected after 4 h and 24 h of heat stress. Collected leaves were immediately frozen in liquid nitrogen and stored at -80°C until further use.

RCA analysis

RCA was analysed by using 1-D SDS-PAGE and immunoblotting (Ristic *et al.*, 2008). Leaf proteins were extracted in 10% (w/v) trichloroacetic acid (TCA) according to Wu and Wang (1984), and protein concentration was determined by using the RC DC Protein Assay (Bio-Rad, Hercules, CA). Extracted proteins were separated on 10% polyacrylamide gels. Equal amounts of protein were loaded on the gels. Following electrophoresis, proteins were transferred to a PVDF membrane (Bio-Rad, CA). Blots were probed for RCA with tobacco anti-RCA antibody (maize and wheat RCA blots, Feller *et al.*, 1998), an antibody that was raised (Sigma Genosys Biotechnologies, The Woodlands, TX) by using a synthetic peptide of amino acids (CGALRRVYD-DEVK) deduced from the nucleotide sequence of wheat RCA cDNA (wheat blots, Genbank Accession Number: AF251264) or *Arabidopsis* anti-RCA antibody (*Arabidopsis* blots, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Expression levels of RCA were estimated by determining band volume with Quantity One software (Bio-Rad, CA). Data on band volumes from two replicate plants were averaged and related to plant yield [total shoot dry mass (determined in the same experiment used to investigate expression levels of RCA) and grain yield (determined over a period of 11 years under field conditions)].

Data analysis

Statistical comparison of experimental duplicates on expression levels of RCA (band volumes) was conducted using PROC GLM Procedures in Statistical Analysis System (SAS Institute, 2003). There was no significant differences between the two replicates when cross-reacting RCA band volume in heat-stressed plants are expressed as a percentage of control plants. The replicate data are shown in Supplementary Table 1 at JXB online. Data on expression levels of RCA (RCA band volumes, variable X) were plotted against total shoot dry mass (variable Y) or average grain yield encompassing a period of 11 years (variable Y). Band volumes of each RCA cross-reacting band observed on immunoblots were plotted separately against total shoot dry mass or average grain yield. Correlation analysis with PROC CORR in the Statistical Analysis System (SAS Institute, 2003) was used to quantify the relationship and to test the significance of correlations between RCA band volumes and wheat yield (total shoot dry mass or 11-year-average grain yield). The data of all variables was continuous and normally distributed; therefore, Pearson's correlation coefficient was estimated. The statistical significance of correlation coefficient was tested by using the *P* value.

Results and discussion

RCA expression in wheat

Immunoblots prepared with leaf protein extracts from control plants [grown at $22/17^{\circ}\text{C}$ (day/night)] of 11 European cultivars of winter wheat showed three RCA cross-reacting bands (Fig. 1). The molecular mass of the observed bands was 45–46 kDa, 42 kDa, and 40–41 kDa. Exposure to 7 d of heat stress at $36/30^{\circ}\text{C}$ (day/night) did not seem to affect expression of the 42 kDa RCA but slightly affected expression of the 45–46 kDa and 40–41 kDa RCAs in several cultivars (Fig. 1; see Supplementary Table 1A, B at JXB online). As indicated by band volume, the amount of the 45–46 kDa polypeptide cross-reacting with the anti-RCA antibody appeared lower, relative to the respective control, in heat-stressed plants of cultivars Stepa ($\sim 38\%$), Proteinka ($\sim 29\%$), and Stamena ($\sim 25\%$) and higher in cultivar Ljiljana ($\sim 24\%$). Likewise, the relative amount of the 40–41 kDa polypeptide cross-reacting with the anti-RCA antibody appeared lower, compared with the respective control, in heat-stressed plants of cultivars Proteinka ($\sim 26\%$), Ljiljana ($\sim 23\%$), and Stamena ($\sim 21\%$) and higher in heat-stressed plants of cultivars NS2-4523 ($\sim 89\%$) and Dragana ($\sim 48\%$) (Fig. 1).

The appearance of three cross-reacting RCA bands in 11 cultivars of winter wheat contrasts with previous reports on the number of RCA isoforms. As stated earlier, in most species studied, RCA was found in two isoforms, the longer α (43–47 kDa) and the shorter β (41–42 kDa) (Salvucci *et al.*, 1987). RCA is susceptible to proteolysis during protein extraction from the leaf tissue (Zielinski *et al.*, 1989; Rundle and Zielinski, 1991; Salvucci *et al.*, 1993). To avoid possible degradation of RCA polypeptides, the protein extraction method of Wu and Wang (1984) was used; this has been shown to eliminate protein degradation during protein extraction from the leaf tissue. This method includes several steps starting with freezing the leaf tissue in liquid N_2 , extracting proteins from the frozen leaf tissue directly in 10% TCA, and then solubilizing the precipitated proteins in a sample buffer containing SDS at high temperature. Thus, it is unlikely that RCA underwent degradation during protein extraction in these experiments.

To test further the possibility that the three RCA cross-reacting bands in wheat may be an artefact due to protein degradation, RCA expression was investigated in four additional cultivars of wheat (two spring wheat cultivars and two winter wheat cultivars), five genotypes of maize, and one genotype of *Arabidopsis thaliana*. This time, plants were exposed to different stress treatments to test the possibility that treatment conditions (temperature and duration of heat stress) may have an effect on RCA expression. Wheat was exposed to 40°C for 4 h, maize to 45°C for 4 h, and *Arabidopsis* to 35°C for 4 or 24 h. Following exposure to heat stress, proteins were extracted from both control and heat-stressed plants according to the

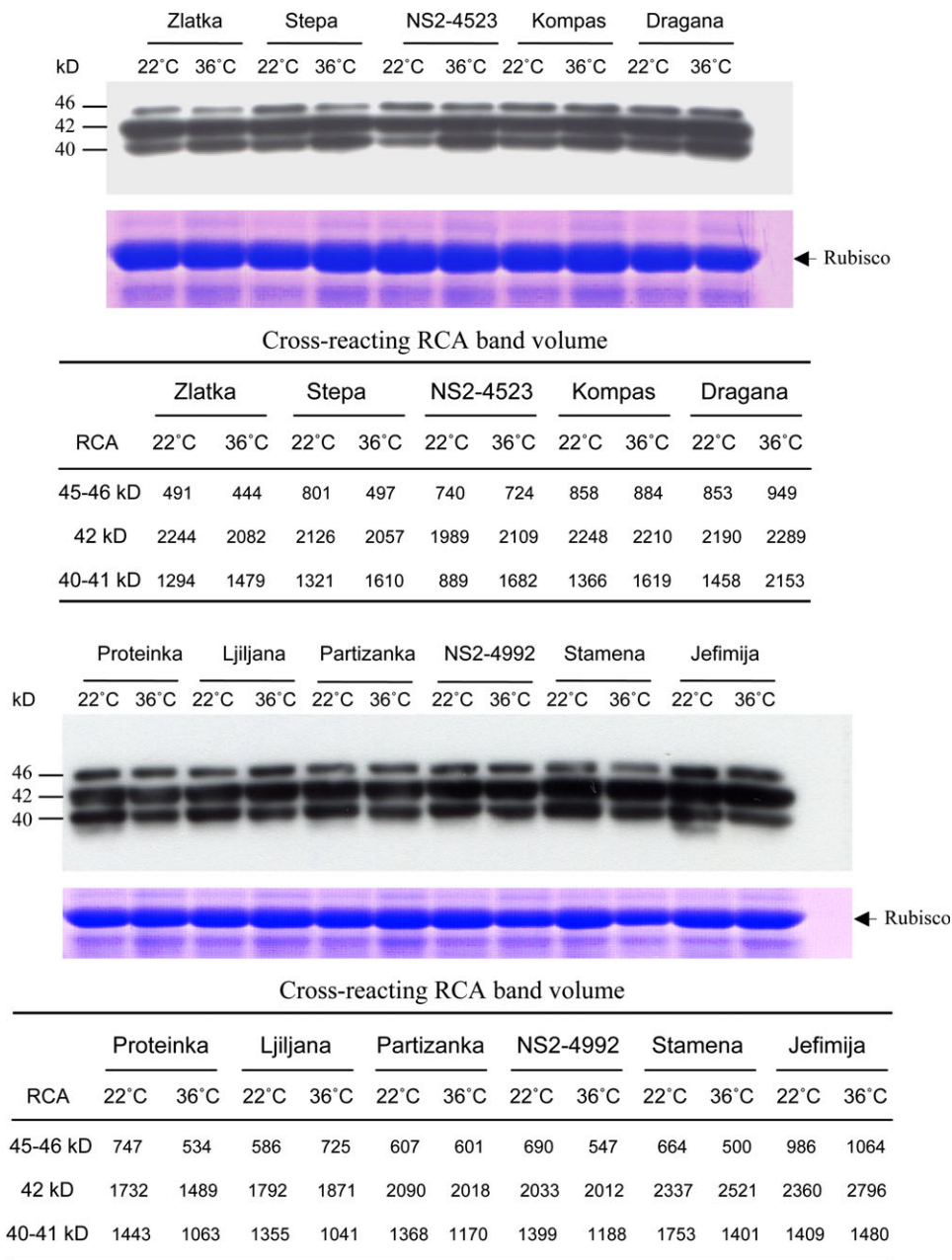


Fig. 1. Immunoblot analysis of leaf protein extracts from control (22 °C) and heat-stressed (36 °C) plants of European cultivars of winter wheat. Plants were exposed to heat stress at the beginning of flowering for 7 d. Proteins were extracted from flag leaves and analysed by using 1-D SDS-PAGE and immunoblotting. Immunoblots were probed with wheat anti-Rubisco activase antibody. An equal amount of protein (15 µg) was loaded in each lane. The Coomassie Brilliant Blue R250 stained gel below each immunoblot shows the Rubisco large subunit (loading control).

extraction method of Wu and Wang (1984) and analysed with 1-D SDS-PAGE and immunoblotting. In all four wheat cultivars, three RCA cross-reacting bands with a molecular mass ranging from 40 kDa to 46 kDa (45–46 kDa, 42 kDa, and 40–41 kDa) were observed on immunoblots of both control and heat-stressed plants (Fig. 2). A brief exposure to heat stress (4 h at 40 °C) appeared to have some effect on RCA expression in some cultivars (Fig. 2; see Supplementary Table 1C at *JXB* online). For example, as indicated by relative band volume, prominent changes in

the amount of 40–41 kDa RCA cross-reacting band (relative to the respective control) were detected in heat-stressed plants of cultivars Seri-82 (~67% decrease) and Karl-92 (~31% increase).

The observation of the effect of prolonged heat stress on RCA expression in mature wheat differs from a previous study that examined expression of this protein in the same species following short-term exposure to heat stress. Law and Crafts-Brandner (2001) studied RCA expression in young, non-flowering plants of wheat following exposure

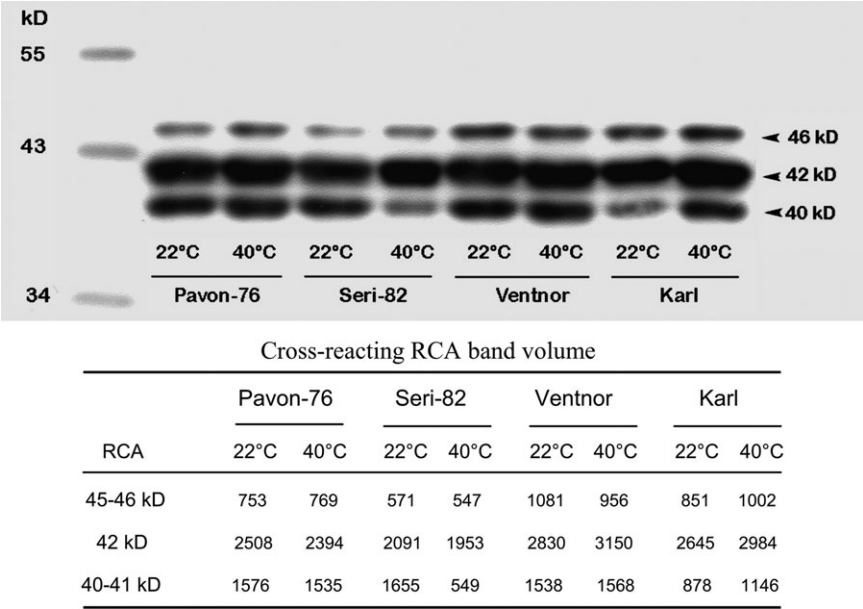


Fig. 2. Immunoblot analysis of leaf protein extracts from control (22 °C) and heat-stressed (40 °C) plants of spring (Seri-82 and Pavon-76) and winter (Ventnor and Karl) wheat cultivars. Mature plants (flowering stage) were exposed to 40 °C for 4 h. Proteins were extracted from flag leaves and analysed by using 1-D SDS-PAGE and immunoblotting. Immunoblots were probed with wheat anti-Rubisco activase antibody. An equal amount of protein (15 µg) was loaded in each lane.

to 24 h or 48 h of heat stress [38/34°C (day/night)]. They noted that extracts from control plants had two RCA cross-reacting bands of 42 kDa and 46 kDa. After 48 h of heat stress, Law and Crafts-Brandner (2001) observed a decrease in abundance of the 46 kDa RCA, an increase in abundance of the 42 kDa RCA, and appearance of a new putative RCA form of 41 kDa. It is possible that differences in RCA expression patterns between the present study and that of Law and Crafts-Brandner (2001) are due to differences in plant age and/or experimental conditions.

The appearance of three RCA cross-reacting polypeptide bands in mature plants of wheat raises an important question. What is the origin of these polypeptides? Are they a product of post-translational modification or alternative splicing or expression of different genes without this regulation or combination of these regulatory mechanisms? The wheat genome is composed of three different sets of chromosomes (Cox, 1998), and it is possible that wheat also has three copies of RCA genes. Further studies are needed to determine the number of RCA genes in wheat and to investigate the origin [mechanism(s) of expression] of three RCA cross-reacting polypeptides.

RCA expression in maize

Experiments with maize revealed an intraspecific qualitative difference in RCA expression. Under control conditions, all five genotypes had two RCA cross-reacting bands of a molecular mass of 43 kDa and 41 kDa (Fig. 3). Following exposure to heat stress (4 h at 45 °C), maize

lines ZPBL 1304, B-73, Zmeftu-wt, and Zmeftu::mum540 synthesized and accumulated a new putative RCA isoform of 45–46 kDa, whereas line ZPL 389 did not (Fig. 3, indicated by arrow). Two of the maize lines, ZPBL 1304 and ZPL 389, have previously been compared for their tolerance to heat stress, with line ZPBL 1304 being described as heat tolerant and ZPL 389 as heat sensitive (Ristic and Cass, 1992, 1993). Interestingly, in the present study, the heat-tolerant line ZPBL 1304 synthesized and accumulated a new putative RCA form of 45–46 kDa, but the heat-sensitive line ZPL 389 did not. It is not known whether the 45–46 kDa RCA cross-reacting band is related to heat-stress tolerance in maize. A previous study showed that, under combined heat and drought stress, the heat-tolerant line ZPBL 1304 displayed higher rates of CO₂ fixation compared with the heat-sensitive line ZPL 389 (Ristic, 1992). It is possible that the heat-induced 45–46 kDa RCA cross-reacting band may be of importance to maize photosynthesis during exposure to high-temperature stress conditions. Further studies are needed to investigate this possibility.

Expression of RCA has been previously studied in maize. Sánchez de Jiménez *et al.* (1995) observed that leaf extracts of 10-d-old seedlings obtained before heat stress had only one 41 kDa RCA band. Under heat-stress conditions (4 h at 45 °C), this band increased in intensity, and a new heavier RCA band of 43 kDa appeared (Sánchez de Jiménez *et al.*, 1995). By comparison, in the present study, two RCA cross-reacting bands were detected in control plants and three RCA-cross-reacting bands were detected in heat-stressed plants. Moreover, the intensity of the constitutively

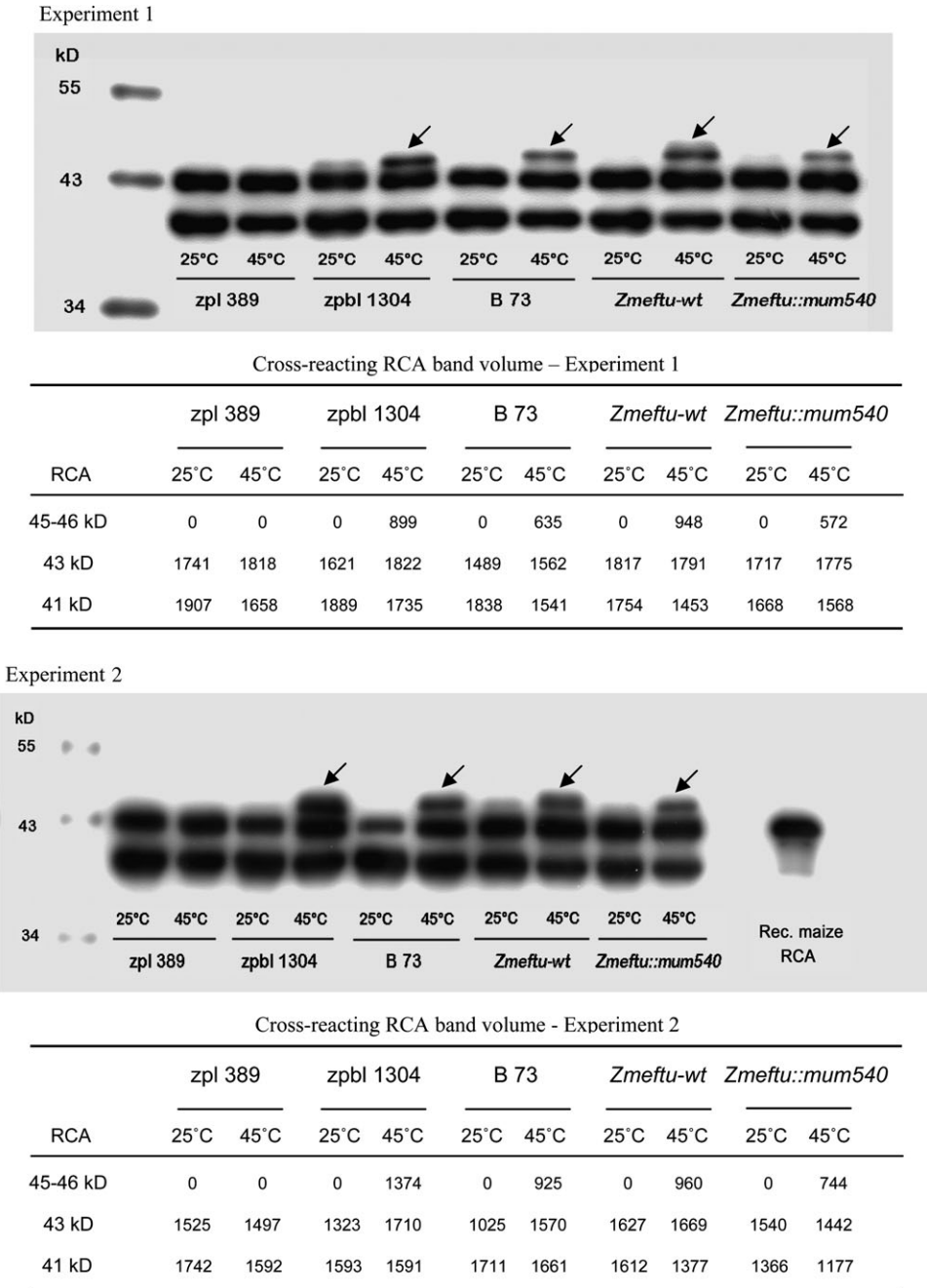


Fig. 3. Immunoblot analysis of leaf protein extracts from 3-week-old control (25 °C) and heat-stressed (45 °C) plants of maize genotypes. Two independent experiments were conducted (Experiments 1 and 2). In each experiment, plants were exposed to 45 °C for 4 h. Proteins were extracted from the youngest fully expanded leaves and analysed by using 1-D SDS-PAGE and immunoblotting. Immunoblots were probed with tobacco anti-Rubisco activase antibody. An equal amount of protein (15 µg) was loaded in each lane. The arrow indicates a new, heat-induced, putative RCA isoform of 45–46 kDa. Rec. maize RCA, recombinant maize Rubisco activase. Note: Recombinant maize RCA of 43 kDa (Ristic *et al.* 2007) was loaded as a control to check the molecular mass of maize RCA cross-reacting bands.

expressed bands did not appear to increase following exposure to heat stress (Fig. 3). As in the experiment with wheat, it is possible that differences in RCA expression between this study and that of Sánchez de Jiménez *et al.* (1995) may be due to differences in plant age. The present experiments used 3-week-old maize plants, but Sánchez de

Jiménez *et al.* (1995) investigated RCA expression in 10-d-old seedlings.
RCA expression in Arabidopsis
In contrast to maize and wheat, heat stress did not perceptibly change the levels or pattern of RCA expression

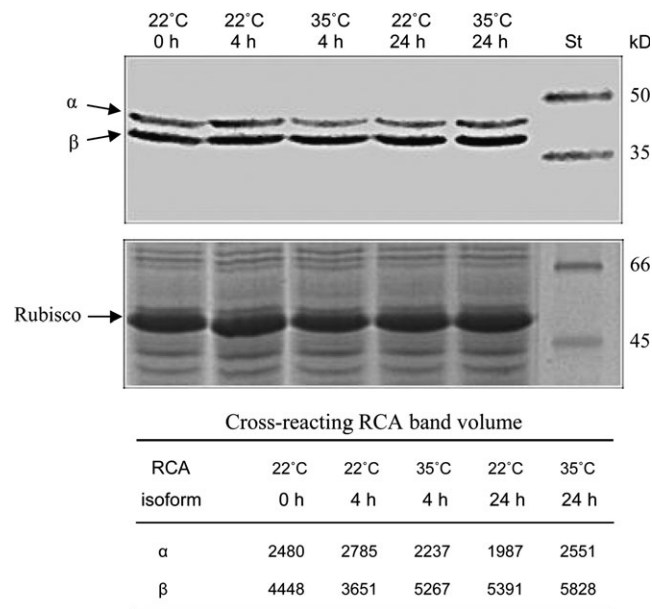


Fig. 4. Immunoblot analysis of leaf protein extracts from control (22 °C) and heat-stressed (35 °C) plants of *Arabidopsis thaliana*. Proteins were extracted from leaves and analysed by using 1-D SDS-PAGE and immunoblotting. Immunoblots were probed with anti-*Arabidopsis* Rubisco activase antibody. An equal amount of protein (10 µg) was loaded in each lane. The Coomassie Brilliant Blue R250 stained gel below the immunoblot shows the Rubisco large subunit (loading control).

in *Arabidopsis*. In this model species, immunoblot analysis of protein extracts revealed two RCA cross-reacting bands in both control and heat-stressed plants (Fig. 4). The two bands of control plants appeared similar to those of heat-stressed plants with respect to volume and intensity (Fig. 4; see Supplementary Table 1D at JXB online). Results from replicated experiments did not indicate consistent differences in expression of either RCA isoform in heat-stressed *Arabidopsis* tissue relative to control samples, and refute the hypothesis that induction or synthesis of new RCA isoforms is a universal phenomenon in higher plants (Law *et al.*, 2001).

Furthermore, the combined experiments with wheat, maize, and *Arabidopsis* did not support the hypothesis that the observed three RCA cross-reacting bands in wheat are due to proteolysis of RCA. If RCA was being degraded during protein extraction, one might expect to see evidence of RCA degradation in all three species. However, this was not the case. In *Arabidopsis*, two RCA cross-reacting bands were detected under both control and heat-stress conditions, and in maize, two bands were seen under control and three under heat-stress conditions. The new band that appeared in maize under heat-stress conditions had a molecular mass that was higher than the molecular mass of the two constitutive RCA bands (Fig. 3). This argues against the possibility of RCA degradation during protein extraction from leaf tissue. One may postulate that RCA from different species may differ in susceptibility to proteolysis or be subject to degradation by different sets of proteolytic

enzymes. The researchers do not completely rule out these possibilities but are unaware of any reports indicating interspecific or intraspecific differences in RCA susceptibility to proteolytic degradation.

RCA expression levels and plant productivity in wheat

Exposure to 16 d of heat stress had a profound negative effect on plant productivity in all 11 European cultivars of winter wheat, as indicated by a significant reduction in total shoot dry mass (Fig. 5A). The possible relationship between wheat productivity and the abundance of RCA was investigated by plotting the band volume of each individual RCA cross-reacting band (variable X) against the average total shoot dry mass (variable Y). The RCA band volume was obtained from immunoblots prepared with protein samples from heat-stressed plants; protein samples from all 11 cultivars were loaded on the same gel (Fig. 5C). No significant correlation between the two variables was seen in plants grown under control conditions (data not shown). Also, no significant correlation was seen between plant productivity and the abundance of 40–41 kDa or 42 kDa RCA cross-reacting bands in plants experiencing heat stress (data not shown). However, a significant positive linear correlation ($P < 0.05$) was seen between the 45–46 kDa RCA cross-reacting band and the total shoot dry mass in heat-stressed plants (Fig. 5B).

The possible relationship between the abundance of the 45–46 kDa RCA cross-reacting band and plant productivity was further examined by plotting the 11-year average grain yield (variable Y) (obtained under field conditions from 1996 to 2006) against the volume of the 45–46 kDa RCA cross-reacting band (variable X). This analysis also revealed a significant positive linear correlation ($P = 0.013$) between the abundance of the 45–46 kDa RCA cross-reacting band and plant productivity (Fig. 6A). It should be noted that the wheat cultivars from which data on grain productivity were obtained have repeatedly, every year from 1996 to 2006, experienced supraoptimal temperatures in the field, as indicated by the average daily maximum temperature for the months of May and June, the number of days in which the maximum air temperature exceeded 30 °C (Fig. 6B), and total precipitation (Fig. 6C). The optimum temperature for growth and yield of wheat is in the range of 18–24 °C, and exposure to elevated temperatures of 28–32 °C even for short periods (5–6 d) is stressful for wheat (Paulsen, 1994; Stone and Nicolas, 1994; Mullarkey and Jones, 2000).

The positive correlation between the abundance of 45–46 kDa RCA and plant productivity is in concurrence with Martínez-Barajas *et al.* (1997), who reported that a high-yielding population of maize had higher levels of RCA than a low-yielding population. Thus, results of the present study support the hypothesis that endogenous levels of RCA may play an important role in plant productivity under supra-optimal temperatures, and that heat-mediated adjustment of existing RCA protein levels or the production of novel RCA isoforms may allow crop plants to maintain the high

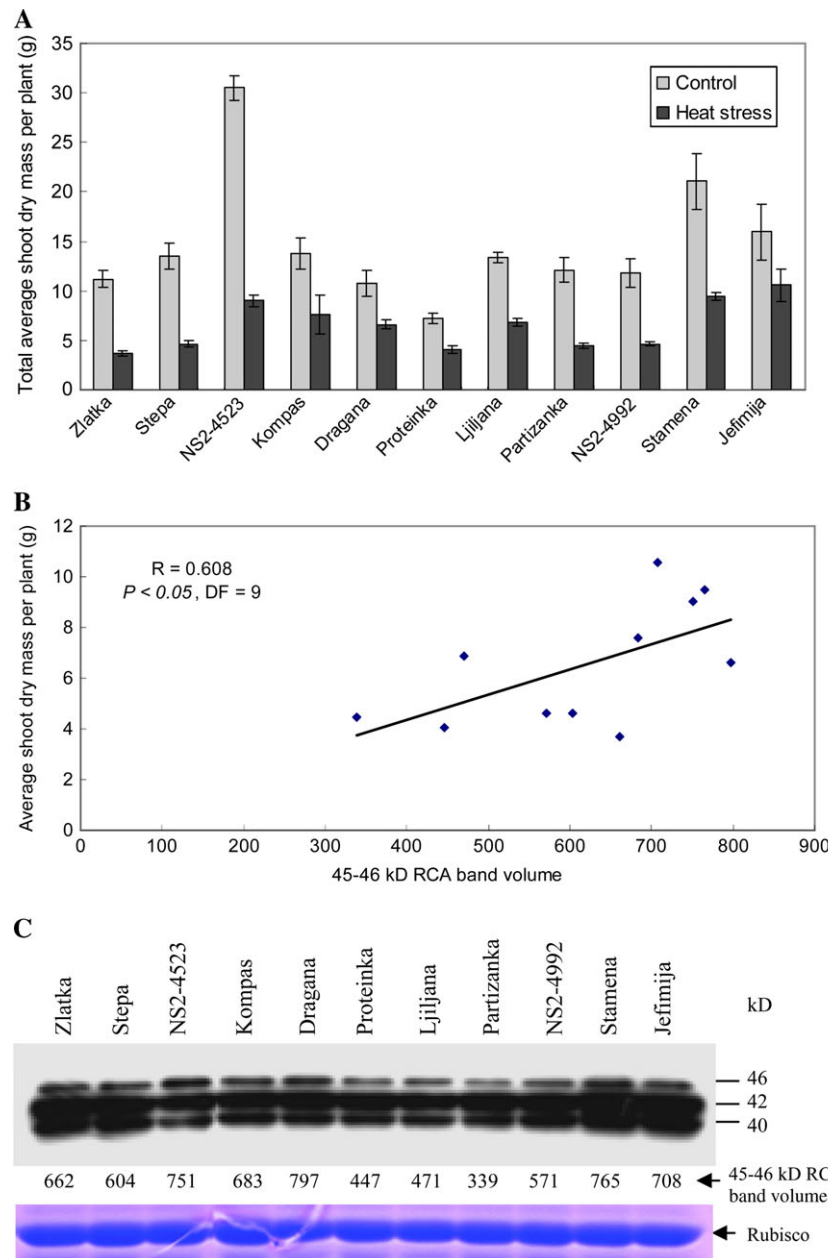


Fig. 5. (A) Total shoot dry mass of European winter wheat cultivars after exposure to heat stress [36/30 °C (day/night)] for 16 d. Bars indicate standard error ($n=13$). (B) Correlation between the 45–46 kDa RCA (Rubisco activase) band volume and total shoot dry mass in heat-stressed plants of 11 European cultivars of winter wheat. Data on RCA band volume were obtained from an immunoblot prepared with protein samples from heat-stressed plants (shown in panel C). (C) Immunoblot analysis of leaf protein extracts from heat-stressed plants [7 d at 36/30 °C (day/night)] of European cultivars of winter wheat. Proteins were extracted from flag leaves and analysed by using 1-D SDS-PAGE and immunoblotting. Immunoblots were probed with wheat anti-Rubisco activase antibody. An equal amount of protein (15 µg) was loaded in each lane. The Coomassie Brilliant Blue R250 stained gel below the immunoblot shows the Rubisco large subunit (loading control). Similar results (trend) on the band volume of 45–46 kDa cross-reacting RCA were obtained in a duplicate blot (see Supplementary Table 1E at JXB online).

levels of photosynthesis necessary to sustain higher yields during periods of heat stress.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Table S1. (A, B) Cross-reacting RCA band volume in European cultivars of winter wheat: replica 2. (C) Cross-reacting RCA band volume in European cultivars of winter wheat: replica 2. (D) Cross-reacting RCA band volume in *Arabidopsis*: replica 2. (E) Cross-reacting RCA band volume in heat-stressed European cultivars of winter wheat: replica 2.+

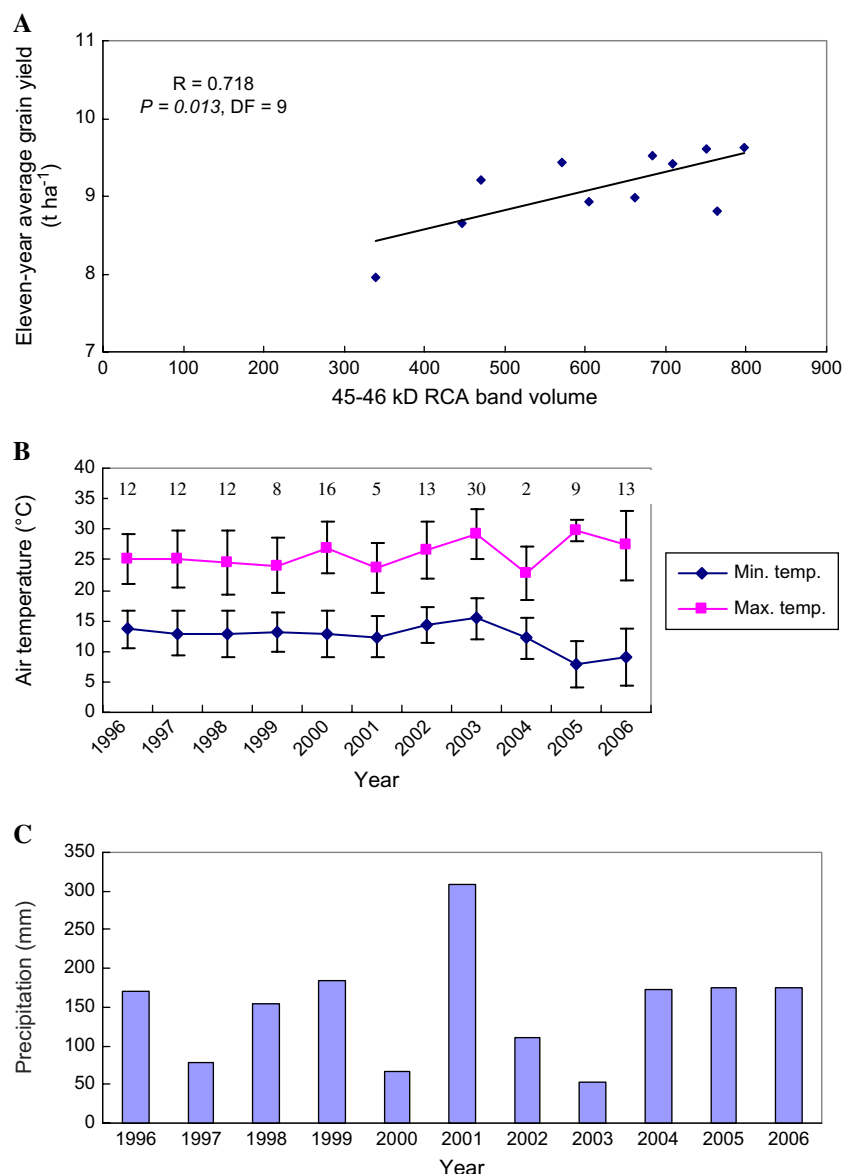


Fig. 6. (A) Correlation between the 45–46 kDa RCA (Rubisco activase) band volume and 11-year-average grain yield (1996–2006) in European cultivars of winter wheat. Data on RCA band volume were obtained from an immunoblot prepared with protein samples from heat-stressed [7 d at 36/30 °C (day/night)] plants (shown in Fig. 5C). For more information on immunoblot preparation, see the legend of Fig. 5 or the Materials and methods. (B) Average minimum and maximum daily temperature for the months of May and June over the 11-year time period from 1996 to 2006. Bars indicate standard deviation. Numbers above the data points indicate number of days for the months of May and June when the maximum daily temperature was ≥ 30 °C. (C) Total precipitation for the months of May and June from 1996 to 2006.

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